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Research Paper: The fate of neural stem/progenitor cells exposed to different concentrations of methylprednisolone

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ABSTRACT

The fate of neural stem/progenitor cells (NS/PCs) is very important in combination therapies for spinal cord injury (SCI). Methylprednisolone (MP) is the main anti-inflammatory drug currently used in the acute phase of SCI. The present study evaluated the differentiation patterns of NS/PCs following exposure to different concentrations of MP.

NS/PCs were isolated from ganglionic eminence of mouse embryos. After the second passage, the obtained cells were treated with 0, 5, 10, 15 and 20 µg/ml of MP. The differentiation of the cells in to immature neuroblasts, mature neurons, astrocytes, and oligodendrocytes was assessedusing immunofluorescence assay.

Exposure of the NS/PCs to different concentrations of MP increased the production of astrocytes, neuroblasts, and oligodendrocytes, Whileit didn't have any considerable effect on the production of more mature neurons.

In conclusion, MP can change the fate of NS/PCs toward the generation of astrocytes and oligodendrocytes. This effect should be considered in MP+NS/PCs combination therapy strategies.

1. Introduction

Spinal cord injury (SCI) as a debilitating condition with high incidence in the young population poses substantial financial and physical expenses to both society and the individuals. A review of studies from different countries has reported the annual incidence range of 11.5 to 57.8 cases per million people in various regions with a variety of etiologies such as car accidence, falls and violence-related injuries such as gunshot [1]. Responses after SCI appear in three phases, including the acute, secondary, and chronic stages. The acute phase -limited to the first few days- is characterized by mechanical neural damage necrosis or cell death,

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hemorrhage and localized edema. The expansion of cellular death and edema, as well as lipid peroxidation, and the production of free-radical induced by cytotoxic concentrations of excitatory amino acids occur as the secondary injuries lasting for minutes to weeks.

Methylprednisolone (MP),as a steroid, has been suggested as the most successful agent for edema control and the reduction of inflammatory responses in the acute phase of SCI during the last decade [2]. Although some improvement in motor recovery has been reportedfollowing the administration of high doses of MP in the early phase of SCI, its efficacy is controversial [3]. The development of acute corticosteroid myopathy [4] and increasinginfection are some reported side effects of the current protocol of systemic MP administration [3]. Given the increasing evidence regarding the undesirable outcomes of MP, presenting a different route of administration such as local injection is a crucial issue.

Stem cell-based treatments including transplantation of embryonic/adult cells or mobilization of endogenous cells are among the current approaches developed for the recovery of SCI [5]. Growth factor production by neural stem/progenitor cells (NS/PCs)including NGF, BDNF, and GDNF has resulted in regrowth of axons [6]. Furthermore, sensory-motor improvement have been achieved by grafting NS/PCsderived from the human fetal spinal cord to the rat model of SCI [7].

Considering the application of MP as a pharmaceutical treatment for neurological dysfunctions and the increasing interest in stem cell-based strategies for recovery of neuronal damages, the effects of MP on stem cell behaviors have been evaluated in some studies. Improved neurological deficits and increased migration of neurons in the striatum of rats with cerebral ischemia were observed following MP administration. However, cell proliferation was not affected [8].Besides, oligodendrocytes, unlike neurons, survived better in the presence of MP in rats with SCI [9]. Conversely, the exposure of endogenous neural progenitor cells collected from the spinal cord of rats to MP has resulted in lower proliferation as well as altered differentiation capacity of cells

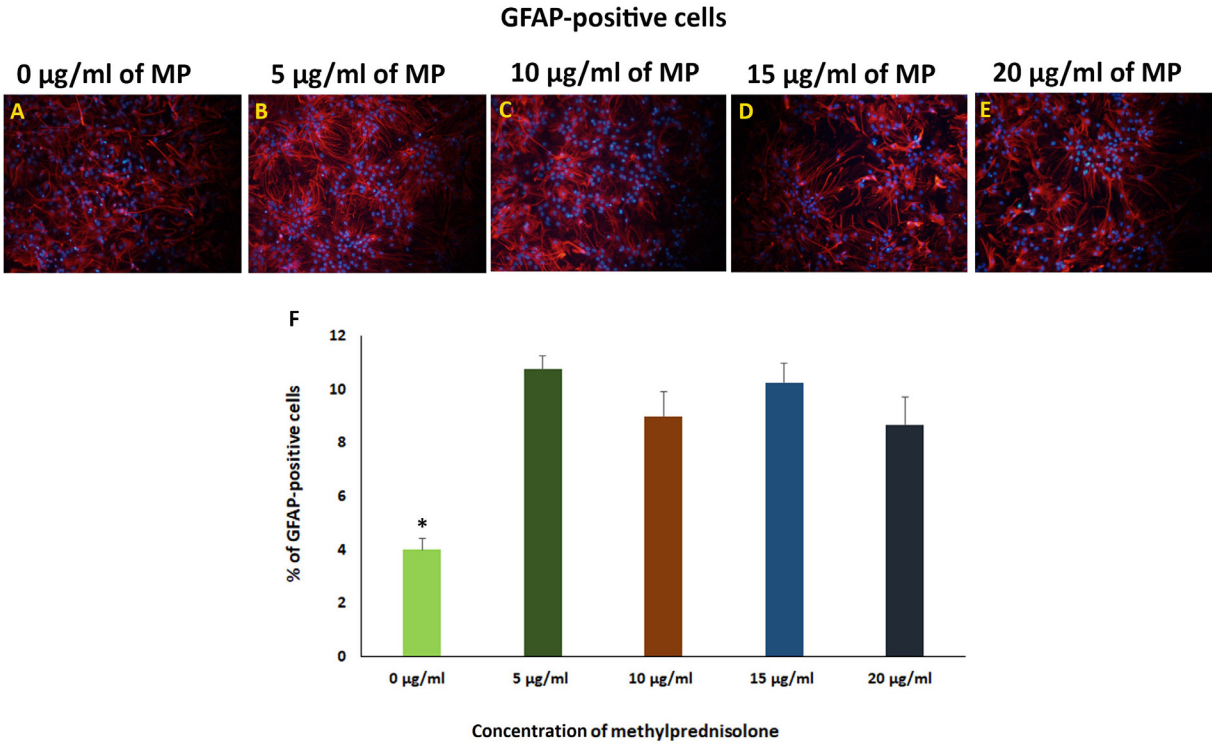

Figure 1. The effect of MP on the percentage of GFAP-positive cells (A-E). MP significantly increased the percentage of astrocytes in all treated groups, however, no differences were found between MP-treated groups in terms of GFAP-positive cells (F). Magnification: 200 x. *: P<0.05 vs the MP-treated groups.

in vitro [10]. Due to these discrepancies and the importance of differentiation of NS/PCs [11], the present study was aimed to evaluate the effects of different concentrations of MP on the differentiation capacity of NS/PCs into neurons and glial cells in vitro.

2. Materials and methods

Isolation and culture of NS/PCs

A 13.5-day old embryo of mice was used for harvesting ganglionic eminence tissue. After mechanical cutting of the specimen, enzymatic dissociation was done by 0.05% Trypsin/EDTA (Invitrogen,USA) for 5 min at 37◦C. Afterward, the activity of the enzyme was inhibited by soybean trypsin inhibitor (Sigma, USA). Next, the cells were cultured in neurosphere medium consisted of Dulbecco's modified Eagle's medium/F12 (Invitrogen, USA), N2 supplement (1%, Invitrogen, USA), B27 supplement (2%, Invitrogen, USA), 1% penicillin/streptomycin, glutamax (1%, Invitrogen, USA) and 20 ng/ml epidermal growth factor (EGF; Miltenybiotech, Germany) at 37◦C and 5% CO2. In the primary culture, the cells were proliferated as free-floating clusters (neurospheres). Every 7 days, the mature spheres were sub-cultured. The cells obtained from the second passage were utilize for the rest of the study.

Study design

The NS/PCs were cultured in differentiation medium (neurosphere medium without EGF and with 5% fetal bovine serum) and exposed to 0, 5, 10, 15 and 20 µg/ml of MP for 7 days.

Cell differentiation assay

The immunofluorescence assay was performed for assessing the fate of NS/PCs in the experimental and control groups. After washing with phosphate buffer saline (PBS) and blocking with 5% normal goat serum (NGS) in PBS, primary antibodies including rabbit anti-doublecortin (for

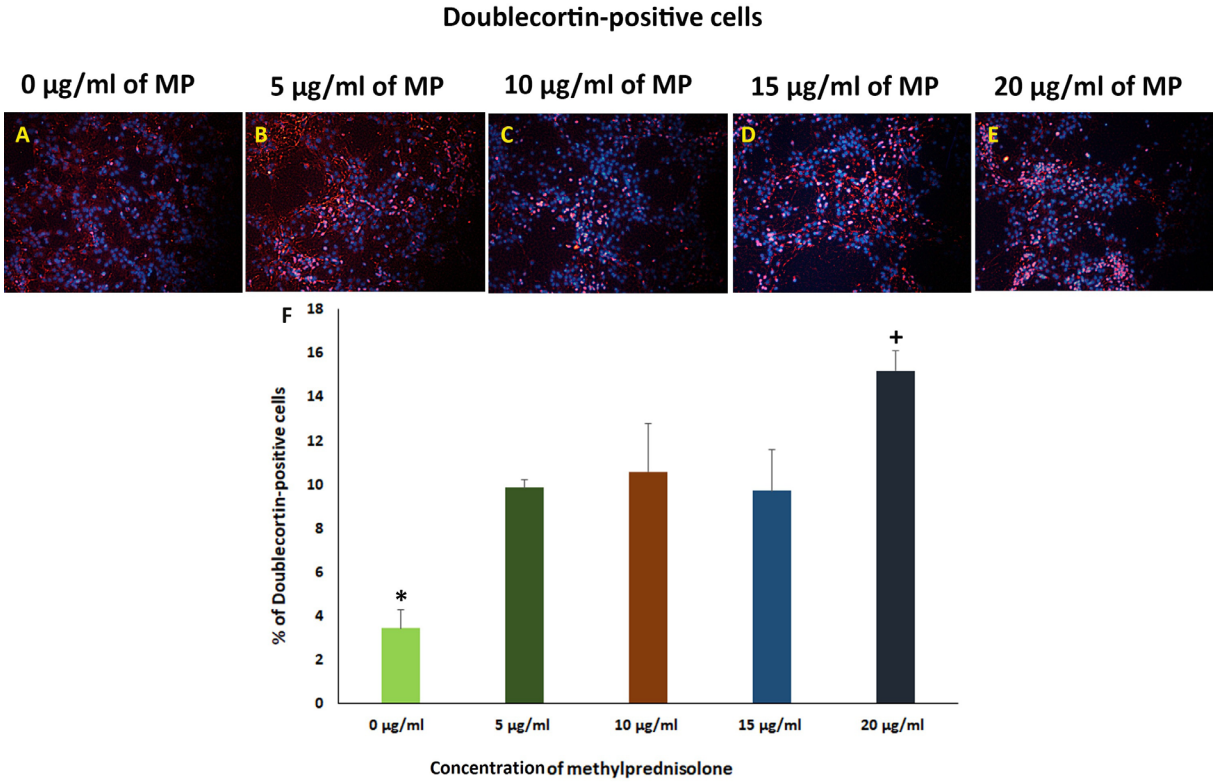

Figure 2. The effect of MP on the percentage of doublecortin-positive cells. The expression of doublecortin is shown in different groups (A-E). MP-treated groups presented significantly more doublecortin-positive cells compared to the control group. Increasing the dose of MP also resulted in more immature neurons (F). Magnification: 200 x. *: P<0.05 vs the MP-treated groups. +: P<0.05 vs all other groups.

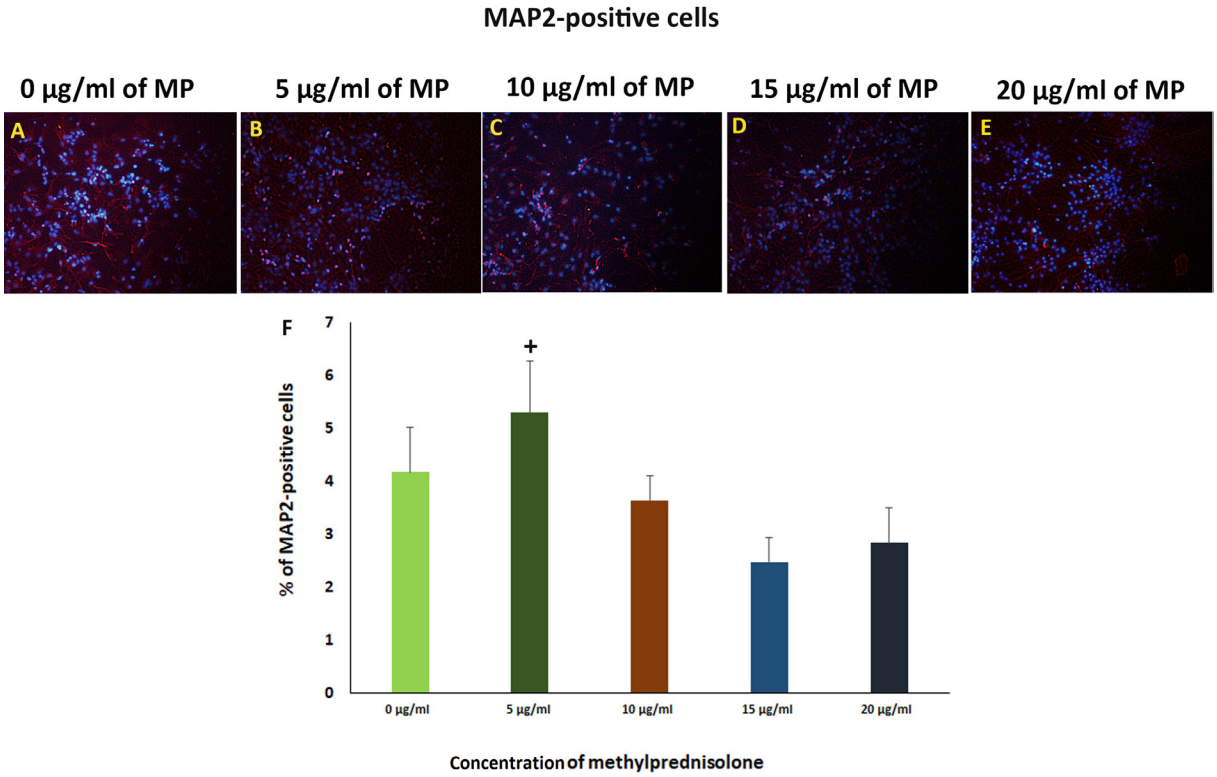

Figure 3. The effect of MP on the percentage of MAP2-positive cells. The expression of MAP2 marker was checked for identifying neurons after the treatment of the cells with different doses of MP (A-E). 5 µg/ml of MP resulted in the highest percentage of MAP2-positive cells (F). Magnification: 200 x. +: P<0.05 vs the 15 and 20 µg/ml of MP.

detection of neuroblasts, 1:200; Santa Cruz, Germany), rabbit anti-MAP2 (as a marker for mature neurons,1:100; Millipore, Germany), rabbit anti-GFAP (as a marker for astrocytes, 1:150, Millipore, Germany), and rabbit anti-Olig 2 (as a marker for oligodendrocytes, 1:50, Millipore, Germany) were applied overnight at 4 °C. Then, gout anti-rabbit fluorescent secondary antibody (Abcam, UK) was used for 2h at room temperature. The nuclei were stained with 40,6-diamidino-2-phenylindole dihydrochloride (DAPI -1:1000, Santa Cruz). As negative control, mouse IgG (Abcam, USA) was replaced by the primary antibody and showed no reactivity. The percentage of immune-positive cells/area was analyzed using a fluorescent microscope (Olympus, Germany).

Statistical analysis

Data is presented as mean ± standard error (SE) of the mean. Prior to statistical analysis, data was checked for normality of distribution. If the distribution of data was normal, one- way analysis of variance (ANOVA) was used forcomparison of the mean of more than two groups. Otherwise, the Kruskal-Wallis test (KWT) was performed as a nonparametric test. P<0.05 was considered statistically significant.

3. Results

Effect of MP on differentiation of NS/PCs to astrocytes

Our results showed that the MP promoted the differentiation of NS/PCs into astrocytes. The percentages of GFAP-positive cells in the MP-treated groups increased by 5-7 % compared to the control group (P< 001, Fig.1). However, increasing the concentration of MP didn't considerably influence the expression of GFAP.

Effect of MP on differentiation of NS/PCs to neuroblasts

Doublecortin- a marker of immature neurons- was also examined in our study. MP increased the

expression of *doublecortin by at least* 6% compared to untreated cells. However, the highest increase was observed in the group treated with 20 µg/ml of MP by about 12% compared to the controlgroup (P<0.05; Fig. 2).

Effect of MP ondifferentiation of NS/PCs to neurons

Differentiation ofNS/PCs to neurons was examined in this study by evaluating the expression of MAP2 marker. The highest percentage of MAP2-positive cells (5.3%) were observed in the group treated with 5 µg/ml of MP which didn't significantly differ from that of control group and concentration 10 (P>0.5) but was considerably higher than concentrations 15 and 20 µg/ml of MP (P<0.5, Fig 3).

Effect of MP on differentiation of NS/PCs to oligodendrocytes

valuation of oligodendrocyte transcription factor 2 (Olig2) in this study showed that all concentrations of MP except concentration 10 significantly and almost equally promoted the differentiation of NS/PCs into Olig2-positive cells compared to the control group (P<0.5, Fig 4).

4. Discussion

In this study, the effect of different concentrations of MP on the differentiation of NS/PCs was evaluated. The percentages of cells differentiated into astrocytes, oligodendrocytes, and neuroblasts increased considerably following exposure to MP. However, different concentrations of MP didn't have any significant effect on the production of mature neurons from NS/PCs.

The CNS is composed of different cell populations, principally neurons and glial cells. Astrocytes and oligodendrocytes are the main glial cells with many essential roles in healthy CNS [12]. Astrocytes are one of the important cellular components found almost in the entire CNS with a ratio of 1.4:1 to neurons in the human cortex. Evidence shows that they have important roles in

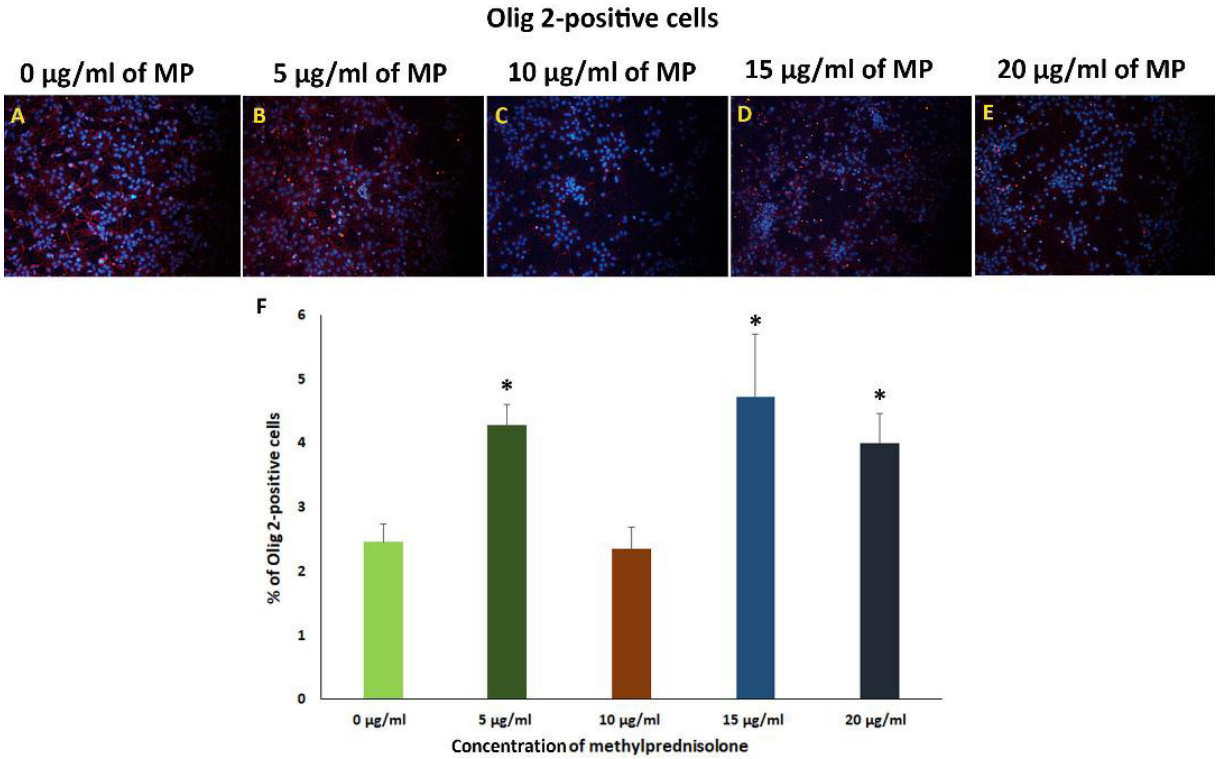

Figure 4. the effect of MP on the differentiation of NS/PCs to oligodendrocytes. The Olig2-positive cells are shown in groups treated with different doses of MP (A-E). Higher percentages of oligodendrocytes, significantly differed from the control group, were observed in the MP-treated groups except dose 10 (F). Magnification: 200 x. *: P<0.05 vs the 0 and 10 µg/ml of MP.

a variety of functions including blood flow regulation, metabolism, transmitter homeostasis, and synaptic transmission [13]. Furthermore, following injury, the astrocytes change to an activated form called reactive astrocytes that contribute to the preservation of tissue integrity.The proliferation of microglia and hypertrophy of astrocytes also results in a phenomenon known as reactive gliosis which has both beneficial and detrimental effects on the surrounding neurons and axonal repair [12]. Although the adverse effects of glial scar formation on axonal regeneration at the site of injury after a brain or spinal trauma has been well documented, recent evidence introduces some neuroprotective functions for reactive astrocytes [13]. In addition to their roles in the protection of CNS from toxic substances, they are involved in endogenous neuroprotection by the secretion of neurotrophic factors including BDNF, NGF, and FGF-2 that potentiate them to contribute to healing the damaged tissue [14]. Based on our results, MP can be used in protocols in which obtaining more astrocytes from NS/PCs is considered.

Doublecortin, known as a microtubule-associated protein, is expressed mainly in migrating neurons not only during development but also in special conditions in adulthood such as injury-induced neurogenesis [15]. The role of *doublecortin* in the radial migration of cortical interneurons has been confirmed by observation of impaired migration of these cells following inactivation of doublecortin in the ganglionic eminences of rat brains [16]. Based on the present study, MP induces the differentiation toward genesis of doublecortin and it can be used when the migration of cells is considered.

Demyelination started within hours after spinal cord injury has also been reported in both animal and human studies. Progressive demyelination primarily due to myelin breakdown or oligodendrocyte apoptosis and necrosis substantially leads to motor dysfunction after many CNS disorders [17]. Oligodendrocytes mostly located in white matter are primarily responsible for myelin formation, an essential parameter for the mature CNS to have a normal function [18]. It is thought that the loss of axonal support provided by oligodendrocyte probably makes the axons more susceptible to degeneration. Accordingly, rapid remyelination of unsupported axons provides them a chance to spare from the subsequent deficits that occur as secondary damages in the following days after SCI [19]. A significant enhanced axonal remyelination resulted from the differentiation of NPCs into oligodendrocyte progenitor cells (OPCs) has led to improved motor and sensory function. Further, increased myelination and decreased lesion size following Olig2 transplantation showed better locomotion recovery [7]. Our results showed that we can use MP to promote the production of oligodendrocytes from NS/PCs by most concentrations of MP.

Microtubule-associated proteins (MAP) 2, which is mainly but not exclusively expressed in neurons, belongs to the structural MAPs family. This protein contains two isoforms, MAP2c and MAP2a, each expressed in a different stage of neuronal development [20]. The possible involvement of MAP2 in some neuron-specific processes arises from its localization along microtubules in differentiated neurons which makes it probably an efficient agent for polymerizing or stabilizing the microtubule andthereby promotes the formation of axons and dendrites. It is also likely to contribute to axonal transport by providing microtubules' linkage to other proteins [21].
Several strategies, however, at the preclinical stages, are being applied to stimulate the regeneration and repair of the injured spinal cord such asmanipulation of the environment for regrowth of injured nerves or the replacement of lost cells [5]. The capacity of differentiation to several cell lineagesandpresence in the spinal cordmake the neural stem cell a potential choice for cell replacement therapy after SCI [22]. The reduced proliferation of neural stem cells treated with MP has beenpreviously shown in some studies [10, 23, 24]. The differentiation of neural stem cells also altered by 10 µg/ml of MP, so that fewer astrocytes but more MAP2-positive cellswere observed in the MP-treated group compared with untreated cells in the study by Wang et al [10],. In contrary to these results, our findings showed that the differentiation of NS/PCs into astrocytes was significantly promoted by MP. In addition, the MAP2-positive cells didn't increase following MP treatment. Another study reported that the administration of MP to the rats with cerebral ischemia resulted in significantly increased

GFAP-positive, MAP2-positive and DCX-positive cells which is somehow in accordance with our findings [8].

In conclusion, MP increased the production of oligodendrocytes and astrocytes. Based on the pathological hallmarks of SCI,replacement of damaged neurons,enhancingremyelination with the help of oligodendrocytes, andcontrollingastrogliosis are the main goals in regenerative strategies. In this sense, MP can help remyelination by increasing the production of oligodendrocytes,however, the increased astrocyte production canhave detrimental effects. So, more complementary in-vivo studies using MP+NS/PCs combination therapies are recommended for future investigations.

Ethical Considerations

Compliance with ethical guidelines

All procedures were performed in accordance with the institutional guidelines of Shiraz University of Medical Sciences for animal care and use.

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Authors contributions

Conceptualization, Author names [HA]; Methodology, Author names [HA]; Investigation, Author names [FS, ZZ]; Writing – Original Draft, Author names [FS, ZZ]; Writing – Review & Editing, Author names [all author]; Funding Acquisition, Author names [HA]; Supervision, Author names [HA].

Conflict of interest

There is no conflict of interest.

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